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(54) Title: TRANSFORMATION OF ZYGOTE, EGG OR SPERM CELLS AND RECOVERY OF TRANSFORMED PLANTS FROM ISOLATED EMBRYO SACS			
(57) Abstract			
<p>Methodology is provided for the production of uniformly transformed plants capable of transmitting a foreign gene to progeny by sexual reproduction. A foreign gene is introduced into the zygote in an isolated embryo sac and a transformed plant is recovered. Alternatively, a foreign gene is introduced into an egg cell in an isolated embryo sac, the egg cell is fertilized with an isolated sperm cell and a transformed plant is recovered. Sperm cells may be transformed with a foreign gene, an egg cell in an isolated embryo sac is fertilized with the transformed sperm cells, or nuclei isolated from the transformed sperm cells, and a transgenic plant is recovered. Another method for the production of transgenic plants is transformation of an embryo in an isolated embryo sac. The transgenic plant produced by any one of these methods is homogeneously transformed and capable of transmitting the foreign gene to progeny by sexual reproduction.</p>			
<p style="text-align: center;">Isolation of Maize Embryo Sacs using the Vibratome</p> <p>A) Components</p> <p>B) Method</p>			

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**TRANSFORMATION OF ZYGOTE, EGG OR SPERM CELLS AND
RECOVERY OF TRANSFORMED PLANTS FROM ISOLATED EMBRYO
SACS**

BACKGROUND OF THE INVENTION

1. Field of the Invention

5 The present invention relates to a method of plant transformation in which a foreign gene is introduced into the zygote in an isolated embryo sac and a transgenic plant is recovered. The present invention also relates to a plant-transformation approach whereby a foreign gene
10 is introduced into an egg cell in an isolated embryo sac, the egg cell is fertilized with a sperm cell, and a transgenic plant is recovered. In addition, the present invention relates to plant transformation by introducing a foreign gene into isolated sperm cells, after which a
15 transformed sperm cell, or the nucleus isolated from the transformed sperm cell, is fused with an egg in an isolated embryo sac and a transgenic plant is recovered. These methodologies yield uniformly transformed plants capable of transmitting a foreign gene to progeny.

20

2. Background

Numerous methods for plant transformation have been developed, including biological and physical plant transformation protocols. See, for example, Miki et al.,
25 "Procedures for Introducing Foreign DNA into Plants" in METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY 67-88 (CRC Press, 1993). In addition, expression vectors and *in vitro* culture methods for plant cell or tissue transformation and recovery of plants are available.
30 See, for example, Gruber et al., "Vectors for Plant Transformation," *loc. cit.* at 89-119.

Production of transgenic plants first became routine

through the use of *Agrobacterium*. The host range for *Agrobacterium*-mediated transformation is broad and includes not only dicotyledonous but also monocotyledonous plants such as rice and maize. Hiei et al., *The Plant Journal* 6: 271-282 (1994) and Ishida et al., *Nature Biotechnol.* 14: 745-750 (1996). Several methods of plant transformation, collectively referred to as "direct gene transfer," have been developed as an alternative to *Agrobacterium*-mediated transformation.

A generally applicable method of plant transformation is microprojectile-mediated transformation wherein DNA is carried on the surface of microprojectiles measuring 1 to 4 μ m. The expression vector is introduced into plant tissues with a biolistic device that accelerates the microprojectiles to speeds of 300 to 600 m/s which is sufficient to penetrate plant cell walls and membranes. Sanford et al., *Part. Sci. Technol.* 5: 27 (1987); Sanford, *Trends Biotechnol.* 6: 299 (1988); Sanford, *Physiol. Plant* 79: 206 (1990); Klein et al., *Bio/Technology* 10: 268 (1992).

Another method for physical delivery of DNA to plants is sonication of target cells. Zhang et al., *Bio/Technology* 9: 996 (1991). Alternatively, liposome or spheroplast fusion have been used to introduce expression vectors into plants. Deshayes et al., *EMBO J.* 4: 2731 (1985); Christou et al., *Proc Nat'l Acad. Sci. U.S.A.* 84: 3962 (1987). Direct uptake of DNA into protoplasts using CaCl_2 precipitation, polyvinyl alcohol, poly-L-ornithine or electroporation have also been reported. Hain et al., *Mol. Gen. Genet.* 199: 161 (1985); Draper et al., *Plant Cell Physiol.* 23: 451 (1982); Fromm et al., *Nature* 319: 791 (1986). Electroporation of protoplasts and plant cells in intact tissue is well documented. Luehrsen et al., in *THE MAIZE HANDBOOK* 613-615, Freeling et al. (eds.) (1994); deHalluin et al., *Plant Cell* 4: 1495-1505 (1992); Laursen et al., *Plant Mol. Biol.* 24: 51-61 (1994).

Finally, microinjection of DNA has been studied in

a variety of plant cells including, for example, isolated protoplasts, callus, microspore-derived embryonic cells and apical meristem. Crossway et al., *Mol. Gen. Genet.* 202: 179-185 (1986); Toyoda et al., *Plant Cell Rep.* 7: 293-296 (1988); Neuhaus et al. *Theor. Appl. Genet.* 75: 30-36 (1987); Simmonds et al., *Physiol. Plantarum* 85: 197-206 (1992).

Although the production of gametes and zygotes by plants is well-understood, reproducible methods for in vitro manipulation and transformation of these cells are needed. In most plant species, embryo sacs are deeply enclosed in the sporophytic tissues and therefore the embryo sacs are difficult to manipulate. Microsporocytes and developing pollen grains are also embedded in sporophytic tissues.

Within the immature anther are cavities containing microsporocytes or pollen mother cells. Each mother cell undergoes two successive nuclear divisions to form a tetrad of four microspores. Each of these microspores may develop into a pollen grain. A microspore develops into a pollen grain by a thickening of the spore wall and a division of the microspore nucleus to form a vegetative cell and a generative cell.

Pollination involves the transfer of pollen from anther to stigma. Pollen germinates on the stigma and a pollen tube grows through the style and enters the tip of the ovule through the micropyle. Two male gametes or sperm cells are formed by division of the generative cell of the pollen grain. The sperm cells move through the pollen tube and are emptied into the embryo sac.

Within each ovule is a megasporocyte that undergoes two successive nuclear divisions to produce four megaspores. Three of the megaspores usually disintegrate while the fourth continues to undergo nuclear divisions and forms an eight nucleate embryo sac. The egg and two synergids are found near the micropyle while three nuclei are found on the opposite end of the embryo sac. Two polar nuclei remain in the center of the embryo. One

sperm cell fuses with the egg cell to form the zygote which eventually develops into the embryo and a new plant. The second sperm fuses with the two polar nuclei to form the primary endosperm nucleus which divides many
5 times to form the endosperm.

Methods for the isolation of living embryo sacs have been developed for plant species by using either micromanipulation or enzymatic digestion. Allington, "Micromanipulation of the Unfixed Cereal Embryo Sac," in
10 THE EXPERIMENTAL MANIPULATION OF OVULE TISSUES 39-51 (Longman, New York, 1985); Theunis et al., *Sex Plant Reprod.* 4: 145-154 (1991); Wu et al., *loc. cit.* 6: 171-175 (1993). In vitro manipulation of fertilized embryo sacs frequently results in low viability or production of
15 abnormal embryos. Leduc et al., *loc. cit.* 8: 313-317 (1995).

Many plant transformation systems, especially for cereals, involve callus-based selection protocols and extensive in vitro culturing. These methodologies are
20 time consuming and increase the likelihood that somaclonal variants will arise that exhibit undesirable agronomic characteristics. Use of developmentally organized explants as targets for transformation circumvent time-consuming tissue culture steps but
25 increases the likelihood that chimeric plants are produced.

Targeting gametes, zygotes or early stage embryos in embryo sacs for transformation is a potential solution to these problems. Transformed plants can be rapidly
30 recovered thereby eliminating the need for a prolonged tissue culture step. The method thereby circumvents classical somatic tissue culture. Instead, the method permits recovery of transformed plants through normal embryogenesis. In addition, transformed gametes and
35 zygotes, as well as a high percentage of early stage embryos, will give rise to uniformly transformed plants capable of transmitting the foreign gene to progeny.

A need therefore exists for an efficient method for

production of transgenic plants uniformly transformed with a foreign gene. A need also exists for an efficient method for production of transformed plants capable of transmitting a foreign gene to progeny. A further need exists for a method of isolating embryo sacs that permits zygote development into an embryo which will develop into a normal whole plant. In addition, a need exists for a method for the transformation of gamete cells or zygotes.

10

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method for production of plants that are uniformly transformed with a foreign gene but which does not involve prolonged in vitro culture.

Another object of the present invention is to provide an efficient method for production of transformed plants capable of transmitting a foreign gene to progeny.

These and other objects are achieved, in accordance with one embodiment of the present invention, by the provision of a method for producing a transformed plant, comprising the steps of (a) isolating an embryo sac from a plant; (b) introducing an expression vector carrying a foreign gene into the zygote or embryo in said embryo sac; and then (c) recovering a transformed plant from said zygote or embryo.

In another embodiment of the present invention, step (b) of the method for producing a transformed plant comprises (i) introducing an expression vector carrying said foreign gene into the egg cell of said isolated embryo sac; and then (ii) fertilizing said transformed egg cell with a plant sperm cell isolated from said plant, to produce said zygote or embryo.

In yet another embodiment of the present invention, step (b) comprises fertilizing the egg cell in said isolated embryo sac with a plant sperm cell that contains an expression vector carrying a foreign gene, to produce

said zygote or embryo.

In another embodiment of the present invention, step (b) comprises (i) introducing an expression vector carrying a foreign gene into sperm cells from a plant;
5 (ii) isolating nuclei from said transformed sperm cells; and (iii) fertilizing the egg cell in said isolated embryo sac with said isolated nuclei, to produce said zygote or embryo. The method of the present invention is used to produced transgenic monocots, such as cereals,
10 and most preferably transgenic maize.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1 shows the orientation of ovaries on the specimen blocks prior to sectioning on the Vibratome: (a) top view, (b) front view and (c) side view.

Figure 2 presents the method for isolation of plant embryo sacs using the Vibratome: (a) components and
20 (b) methodology.

Figure 3 presents a map of plasmid PHP3953 which carries a gene encoding β -glucuronidase (GUS) for detection of transformed plant cells.

Figure 4 presents a map of plasmid PHP8088 which
25 carries the GFP gene for detection of transformed plant cells.

Figure 5 presents a map of plasmid PHP8904 which is a pTOK246-based vector with the origin of replication of pBR322, a spectinomycin resistance gene and the T-DNA
30 borders.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

1. Definitions

In the description that follows, a number of terms
5 are used extensively. The following definitions are provided to facilitate understanding of the invention.

An embryo sac is typically an eight-nucleate female gametophyte. The embryo sac arises from the megaspore by successive mitotic divisions.

10 A megaspore is one of the four haploid spores originating from the meiotic division of the diploid megaspore mother cell in the ovary and which gives rise to the megagametophyte.

15 A microspore is one of the four haploid spores originating from the meiotic division of the diploid microspore mother cell in the anther and which gives rise to the pollen grain.

The polar nuclei are two centrally located nuclei in the embryo sac that unite with the second sperm in a
20 triple fusion. In certain seeds the product of this triple fusion develops into the endosperm

A structural gene is a DNA sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic
25 of a specific polypeptide.

A promoter is a DNA sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5-prime region of a gene, proximal to the transcriptional start site of a
30 structural gene. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter. Also, the
35 promoter may be regulated in a tissue-specific or tissue-preferred manner such that it is only active in transcribing the associated coding region in a specific tissue type(s) such as leaves, roots or meristem.

A cytoplasmically localized protein is a protein coded for by a gene which does not include any specific signal for targeting of the protein into any subcellular organelle or compartment or any signals for secretion of the protein. For example, the GFP structural gene operably linked 5-prime to a promoter and to an appropriate 3-prime sequence encodes for a protein compartmentalized in the cytoplasm.

A signal or targeting sequence is a structural peptide domain required for targeting of a given polypeptide to a subcellular organelle, subcellular compartment or secretion from the cell. As used herein, the phrase sequence for subcellular localization is intended to refer collectively to any form of signal, targeting or retention sequence as defined herein. Signal sequences for a polypeptide directed to the chloroplast or mitochondrion are largely localized to the amino-terminus of the polypeptide. Signal sequences for a polypeptide directed to the glyoxysomes and peroxisomes are largely localized to the carboxy-terminal domains of the polypeptide. Accordingly, targeted transport of the GFP protein is accomplished by means of chemically joining in proper reading frame, or operably linking, the nucleotide sequence encoding a signal sequence to the 5-prime and/or 3-prime region of the GFP structural gene.

A mitochondrial targeting sequence facilitates the transport of a protein to a mitochondrial compartment. Typically, the mitochondrial targeting sequence is located at the amino-terminus of a polypeptide.

A secretion targeting sequence targets a polypeptide for export into the extracellular space through the ER. For example, operably linking a nucleotide sequence encoding the barley alpha amylase 1 (BAA) secretory targeting sequence to the 5-prime end of a structural gene targets the encoded protein for export into the extracellular space.

A cell wall targeting sequence targets a polypeptide for export from the cell but the polypeptide is

specifically localized to the cell wall. For example, cell wall localization of a polypeptide is accomplished by operably linking a nucleotide sequence encoding BAA 5-prime, and operably linking a nucleotide sequence encoding a portion of the maize hydroxyproline-rich glycoprotein 3-prime to a gene encoding the polypeptide. Steifel et al., *Plant Cell* 2: 785-793 (1990).

A vacuolar signal sequence facilitates the transport of a protein to the vacuole. For example, vacuolar targeting is accomplished by fusing the BAA secretory signal sequence at the amino-terminus of the protein and a sequence encoding a vacuolar signal sequence to the carboxy-terminus. Transport of a polypeptide to the vacuole is therefore accomplished by means of operably linking a nucleotide sequence encoding BAA 5-prime, and a nucleotide sequence encoding a vacuolar signal sequence 3-prime to a gene encoding a polypeptide. Alternatively, vacuolar targeting is accomplished by constructing a nucleotide sequence comprising in the 5-prime to 3-prime direction nucleotide sequences encoding a vacuole signal sequence, BAA and a polypeptide.

An endoplasmic reticulum retention sequence targets a polypeptide for localization in the lumen of the endoplasmic reticulum. For example, a polypeptide is targeted for retention in the endoplasmic reticulum through the addition of the BAA sequence on the amino-terminus and an endoplasmic reticulum signal sequence on the carboxy-terminus of the polypeptide.

A nuclear targeting sequence facilitates transport of a polypeptide to the nucleus. Typically, the nuclear signal sequence is located at the amino-terminus of a polypeptide. In order to retain the nuclear targeted protein in the nucleus, it may be necessary to increase the molecular weight of the protein by means of fusing an unrelated protein to the carboxy-terminus of the targeted protein. For example, GFP was retained in the nucleus by operably linking a nucleotide sequence encoding a nuclear signal sequence 5-prime and a nucleotide sequence

encoding maize acetolactate synthase 3-prime to a gene encoding a polypeptide.

A peroxisomal targeting sequence facilitates the transport of a polypeptide into the peroxisome. Typically, the peroxisomal signal sequence is a tripeptide located at the carboxy-terminus of a polypeptide.

A chloroplast targeting sequence facilitates the transport of a nuclear encoded protein to a chloroplast compartment. Typically, the chloroplast signal sequence is located at the amino-terminus of a polypeptide. Accordingly, transport of a polypeptide to a chloroplast compartment is accomplished by means of operably linking the nucleotide sequence encoding a chloroplast signal sequence to the 5-prime region of a gene encoding a polypeptide.

An isolated DNA molecule is a DNA that is not integrated in the genomic DNA of an organism. An isolated DNA molecule is a chemically-synthesized DNA molecule that is not integrated in the genomic DNA of an organism. [insert*: what?]

Complementary DNA (cDNA) is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its complementary DNA strand.

The term "expression" refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and the translation of mRNA into one or more polypeptides.

A cloning vector is a DNA molecule, such as a plasmid, cosmid, or bacteriophage, that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain one or a small number

of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

5 An expression vector is a DNA molecule comprising a gene that is expressed in a host cell. Typically, gene expression is placed under the control of certain regulatory elements, including constitutive or inducible promoters, tissue-specific regulatory elements, and enhancers. Such a gene is said to be operably linked to
10 the regulatory elements.

Transformation includes introduction of genetic material into plant cells resulting in chromosomal integration and stable heritability through meiosis. Transformation also includes introduction of genetic
20 material into plant cells in the form of plant viral vectors involving epichromosomal replication and gene expression which may exhibit variable properties with respect to meiotic stability.

A foreign gene refers in the present description to a DNA sequence that is operably linked to at least one heterologous regulatory element. A plant is uniformly transformed with a foreign gene if each somatic cell in the plant carries at least one copy of the gene integrated into the plant chromosome.

30 A recombinant host may be any prokaryotic or eukaryotic cell that contains either a cloning vector or expression vector. This term also includes those prokaryotic or eukaryotic cells that have been genetically engineered to contain the cloned gene(s) in the chromosome or genome of the host cell.

35 A transgenic plant is a plant having one or more plant cells that contain an expression vector.

A green fluorescent protein (GFP), as well as a

functional fragment of a GFP, is capable of producing a green fluorescence. GFP absorbs in the UV to blue range with a peak at 395 nm and emits in the green with a peak at 510 nm. GFP_m is a nucleotide sequence coding for GFP in which the DNA sequence has been modified based on codon preference in maize. The nucleotide sequence of GFP_m is shown in co-pending U.S. patent application serial No. 60/016,345 which is incorporated herein by reference.

2. Overview

Plant embryo sacs are isolated by sectioning ovule tissue. Intact plant embryo sacs that can be utilized for *in vitro* transformation and plant recovery are obtained by serially sectioning ovaries. The thickness of the sections varies from species to species depending on the size of the ovule. For example, the thickness of sections for maize is in the range of 250 to 300 μ m. The isolated ovary sections contain embryo sacs surrounded by nucellus and the ovary wall. The structural integrity of the embryo sacs is maintained by this method of isolation. Intact embryo sacs are contained between thin layers of nucellus and are clearly visible using the stereomicroscope. The egg apparatus is visible, showing the egg cell and the two synergids. In addition, antipodal cells are visible. The sections containing the embryo sacs can be readily manipulated with forceps.

The plant embryo sacs isolated by the method of the present invention are viable and can be cultured *in vitro* to recover normal whole plants. The isolated embryo sacs are visible using a stereomicroscope and development can be monitored daily. Within the first week in culture embryo sacs become enlarged as a result of actively growing endosperm. The endosperm proceeds to grow into a large sphere and between the first and second week in culture a proembryo is visible. Embryo development is similar to *in vivo* developmental patterns and results in an embryo with normal morphology. Such embryos can be

germinated by allowing them to remain attached to the endosperm and maternal tissue or can be rescued and germinated separately.

5 The unfertilized egg cell or zygote contained within the isolated embryo sac is transformed with a foreign gene. Alternatively, an embryo sac containing a later stage in the development of the plant, such as a 2-cell, 4-cell or 8-cell embryo, is transformed with a foreign gene. Yet another alternative is transformation of
10 isolated sperm cells with a foreign gene. The transformed sperm cells are then used to deliver the foreign gene to the egg cell during in vitro fusion to form a zygote.

Transformation of gametes, zygote, embryo or
15 endosperm can be accomplished with any of a variety of methods including microinjection, electroporation, particle bombardment or *Agrobacterium*. For example, sperm cells are isolated and transformed with a foreign gene by electroporation. An isolated embryo sac
20 containing an unfertilized egg is incubated with the transformed sperm cells. Fusion of a transformed sperm cell with the egg cell produces a transformed zygote that is cultured in vitro to recover uniformly transformed plant which is capable of transmitting the foreign gene
25 to progeny produced by sexual reproduction. Alternatively, a nucleus is isolated from a transformed sperm cell and fused with an egg nucleus to produce a transformed zygote that is cultured in vitro to recover a transformed plant.

30 The developing endosperm in isolated embryo sacs can be targeted for transformation, for example by microinjection, in order to evaluate the strength of regulatory sequences, such as a specific promoter, in this tissue. Embryo sacs are isolated from a fertilized
35 plant. Endosperm cells are transformed with a foreign gene and allowed to develop in vitro. The foreign DNA, for example, may be a reporter gene such as GUS operably linked to a promoter to be tested. GUS expression is

assayed or quantified by methods well known to the skilled artisan such as Northern blot, PCR or Western blot techniques. See, for example, Ausubel et al. (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Wiley
5 Interscience 1990). Based on the level of expression of the foreign gene in the endosperm a prediction can be made as to the whether the selected regulatory sequence will drive sufficient expression of the foreign gene to modify the phenotype of the seed.

10 Any plant species can be transformed by the claimed method. A particularly preferred plant for transformation by the claimed method are cereals, such as maize. The method of the present invention can be used to circumvent self-incompatibility or species barriers to
15 sexual reproduction. In a plant that exhibits self-incompatibility, pollen tube growth is inhibited. As a consequence, sperm cells are not delivered to the egg sac. Similarly, interspecific hybrids may not be produced due to incompatibility between pollen and
20 stigma. Fusion of sperm and egg cells in an isolated embryo sac circumvents incompatibility between pollen and stigma. Microinjection can be used to introduce a transformed sperm cell or a nucleus isolated from a sperm cell, into the egg. Techniques known to those skilled in
25 the art, using electrical or chemical stimuli, can be used to promote fusion between sperm and egg. See Kranz et al., *Sexual Plant Reproduction* 4: 12-16 (1991) and Deprez et al., *Plant Cell Reports* 14: 204-209 (1995). Further, combinations of these methods such as
30 microinjection of sperm cells into the embryo sac along with fusion-promoting compounds can be used.

A preferred method for introducing foreign DNA into the zygote contained within the isolated embryo sac is microinjection. Embryo sacs are isolated from the
35 fertilized plant. Foreign DNA, for example a reporter gene such as GUS operably linked to a promoter which drives expression in the embryo or plant, is injected into the zygote, which progresses though normal

embryogeny and develops into a plant. GUS expression is assayed or quantified by methods well known to the skilled artisan such as histochemical or fluorimetric assays, Jefferson et al., *EMBO J.* 6: 3902-3907 (1987), or
5 by techniques such as Northern blot, PCR or Western blot, as described by Ausubel et al., *supra*.

3. Expression Vectors

Expression vectors typically include at least one
10 genetic marker that allows transformed cells to be either recovered by positive genetic selection or screening. However, selectable or screenable markers are not absolutely required in the present invention because gamete, zygote or embryo transformation produces
15 homogeneously transformed T0 plants. Consequently, plants recovered following transformation are simply screened for the transgenic trait.

Alternatively, the expression vector may comprise a selectable or screenable marker. Many of the commonly
20 used positive selectable marker genes for plant transformation were isolated from bacteria and code for enzymes that metabolically detoxify a selective chemical agent which may be an antibiotic or a herbicide. Other positive selective marker genes encode an altered target
25 which is insensitive to the inhibitor.

The most commonly used selectable marker gene for plant transformation is the neomycin phosphotransferase II (*nptII*) gene, isolated from Tn5, which when placed under the control of plant regulatory signals confers
30 resistance to kanamycin. Fraley et al., *Proc. Nat'l Acad. Sci. U.S.A.* 80: 4803 (1983). Another commonly used selectable marker is the hygromycin phosphotransferase gene which confers resistance to the antibiotic hygromycin. Vanden Elzen et al., *Plan Mol. Biol.* 5: 299
35 (1985). Additional positive selectable marker genes of bacterial origin that confer resistance to antibiotics include gentamycin acetyl transferase, streptomycin phosphotransferase, aminoglycoside-3'-adenyl transferase

and the bleomycin resistance determinant. Hayford et al., *Plant Physiol.* 86: 1216 (1988); Jones et al., *Mol. Gen. Genet.* 210: 86 (1987); Svab et al., *Plant Mol. Biol.* 14: 197 (1990); Hille et al., *Plant Mol. Biol.* 7: 171 (1986).

Other positive selectable marker genes for plant transformation are not of bacterial origin. These genes include mouse dihydrofolate reductase, plant 5-enolpyruvylshikimate-3-phosphate synthase and plant acetolactate synthase. Eichholtz et al., *Somatic Cell Mol. Genet.* 13: 67 (1987); Shah et al., *Science* 233: 478 (1986); Charest et al., *Plant Cell Rep.* 8: 643 (1990).

Other common selectable marker genes for plants confer resistance to herbicidal inhibitors of glutamine synthetase. European Patent application No. 0 333 033 to Kumada et al. and U.S. Patent No. 4,975,374 to Goodman et al. disclose nucleotide sequences of glutamine synthetase genes which confer resistance to herbicides such as L-phosphinothricin. The nucleotide sequence of a phosphinothricin-acetyl-transferase gene is provided in European application No. 0 242 246 to Leemans et al. De Greef et al., *Bio/Technology* 7: 61 (1989), describe the production of transgenic plants that express chimeric bar genes coding for phosphinothricin-acetyl-transferase activity.

Another class of marker genes for plant transformation requires screening of presumptively transformed plant cells rather than direct genetic selection of transformed cells for resistance to a toxic substance such as an antibiotic. These genes are particularly useful to quantify or visualize the spatial pattern of expression of a gene in specific tissues and are frequently referred to as reporter genes because they can be fused to a gene or gene regulatory sequence for the investigation of gene expression.

Commonly used genes for screening presumptively transformed cells include β -glucuronidase (GUS), β -galactosidase, luciferase, and chloramphenicol

acetyltransferase. Jefferson, *Plant Mol. Biol. Rep.* 5: 387 (1987); Teeri et al., *EMBO J.* 8: 343 (1989); Koncz et al., *Proc. Nat'l Acad. Sci. U.S.A.* 84: 131 (1987); De Block et al., *EMBO J.* 3: 1681 (1984). Another approach
5 to the identification of relatively rare transformation events has been use of a gene that encodes a dominant constitutive regulator of the *Zea mays* anthocyanin pigmentation pathway. Ludwig et al., *Science* 247: 449 (1990).

10 A gene encoding GFP can also be utilized as a screenable marker. Co-pending U.S. application serial No. 60/016,345, incorporated herein by reference, describes strategies for reducing the cellular toxicity of GFP. For example, the gene encoding GFP can be
15 operably linked to an inducible promoter so that GFP can be transiently expressed and transformed cells identified. Alternatively, the gene encoding GFP can be operably linked to a signal sequence for targeting to an organelle or subcellular compartment for secretion to the
20 apoplast where GFP is not toxic to the cell. Signal or targeting sequences for localization in an organelle or subcellular compartment include, but are not limited to chloroplast, peroxisomal, mitochondrial, cell wall, secretion or endoplasmic reticulum retention sequences.
25 GFP can also be modified to replace hydrophobic amino acids with hydrophilic ones thereby reducing the aggregation of the protein and its potential toxicity. Crameri et al., *Nature Biotechnol.* 14: 315-319 (1996).

Oxidative stress can be ameliorated by transforming
30 cells containing GFP with an enzyme that serves as an oxygen scavenger. Such enzymes are well known in the art. For example, genes encoding the enzyme superoxide dismutase (SOD) may protect against the primary oxidative stresses associated with GFP fluorescence. Balzan et
35 al., *Proc. Nat Acad. Sci.* 92: 4219-4223 (1995). Other enzymes involved in the oxidative stress response, such as ascorbate peroxidase or glutathione S-transferase, could help mitigate secondary effects in the cell.

Conklin, *Plant Physiol.* 109: 203-212 (1995).

More specifically, oxidative stress can be ameliorated by transforming cells with a DNA construct carrying genes encoding GFP and SOD. Alternatively, the gene encoding GFP can be fused in frame to the gene encoding SOD. Cells transformed with this DNA construct produce a fusion protein that cause the cells to fluoresce in the presence of UV-blue light and express SOD activity.

The toxicity of GFP in transformed plants can be eliminated by excising the screenable marker gene following detection of transformed cells or sectors. The FLP/FRT system is used in conjunction with GFPm as a visible viable marker for FRT excision. The FLP/FRT system has been demonstrated in maize suspension cells using GUS expression as an indicator of FRT excision. Lysnik et al., *NAR* 21: 969-975 (1993). For example, plant cells are bombarded with a DNA construct containing the GFP gene flanked by FRT sequences as well as a foreign or agronomic gene of interest. The GFP gene may be operably linked to a constitutive promoter or an inducible promoter. In addition, the GFP gene may be operably linked to a signal sequence. Stable transformants are detected by means of screening for GFP.

Transgenic cells are placed on a medium and bombarded a second time with a FLP recombinase construct. Cells are monitored periodically under UV to blue illumination to detect loss of GFP expression. Cells that no longer express GFP are recovered and analyzed for expression of the foreign or agronomic gene. Agronomically useful transgenic plants are thereby produced that do not contain a marker gene.

The sensitivity of the screening method can be further increased by placing two marker genes between the FRT sequences. For example, cells are bombarded with a DNA construct containing the bar gene encoding phosphinothricin acetyl transferase (PAT) and GFP genes flanked by FRT sequences and a foreign or agronomic gene.

Stable transformants are recovered on bialaphos-containing medium and positive GFP-expression is confirmed. The transformed cells are then bombarded with FLP. The cells are then grown for 2-6 weeks with no selection until clear GFP-null sectors can be identified. These sectors can be transferred onto bialaphos/chlorophenol red multiwell test plates to confirm bialaphos sensitivity (i.e., within 3 to 5 days). Cells that no longer express GFP or PAT are recovered and analyzed for expression of the foreign or agronomic gene. This permits recovery of agronomically useful transformants without any marker genes in the final product.

Likewise, the Ac/Ds system of maize can also be used in transgenic plants to excise the screenable marker gene that is transformed together with a foreign or agronomic gene. Mobilization of Ac and/or Ds has been demonstrated in diverse plants such as tomato, tobacco and *Arabidopsis*. Yoder et al., in TOMATO TECHNOLOGY 189-198 (Alan R. Liss, Inc. 1987); Yoder et al., U.S. patent No. 5,225,341; Baker et al., *EMBO J* 6: 1547-1554 (1987); Lawson et al. *Mol. Gen. Genet.* 245: 608-615 (1994). Likewise, the cre/lox recombinase system from bacteriophage P1 could also be used in conjunction with GFP. Excision of transgenes in plants using the cre/lox system was first demonstrated in tobacco. Odell et al., *Mol. Gen. Genet.* 223: 369-378 (1990); Dale & Ow, *Proc. Nat'l Acad. Sci. U.S.A.* 88: 10558-10562 (1991). Similar to the FLP and Ac systems described above, GFP expression provides an efficient, easily scorable phenotype for monitoring excision.

GFP is detected in transformed plant cells by conventional fluorescence detection methods. The transformed cells or tissue are screened for the presence of GFP protein by means of illuminating the cells with UV to blue light and screening for the presence of green fluorescence.

Compound and dissecting microscopes are fitted with

appropriate filter combinations for fluorescent protein excitation. Illumination with UV-blue light is required for visualization (excitation around the absorption maximum of 395 nm or around the minor peak at approximately 475 nm for GFP. A hand held lamp for benchtop work also permits good visualization. Cut-off filters or bandpass filters between the fluorescing tissue and the viewer (i.e. around the intermediate objective or the eyepieces of the microscope, or hand-held in front of the eyes if working on the benchtop) greatly reduces background autofluorescence from the tissue. This cut-off or bandpass filter permits light between 500-550 nm to reach the viewer. Useful filters and wavelengths are not restricted to those described above, and further generic description of the optical characteristics of this system are available. Heim et al., *Currently Biology* 6: 178-182 (1996).

An expression vector is constructed wherein a DNA sequence encoding a foreign protein is operably linked to DNA sequences that regulate gene expression. See, for example, Gruber et al. (1993), *supra*. The gene encoding a foreign protein may be operably linked to a constitutive promoter such as the *nos* or CaMV 35S promoters. An, *Plant Physiol.* 81: 86 (1986); Kay et al., *Science* 236: 1299 (1987). Alternatively, the foreign gene is operably linked to a regulatable promoter such as heat shock promoters, hormone-inducible promoter or light-inducible promoters associated with the small subunit of the RuBP carboxylase and LHCP gene families. Ainely et al., *Plant Mol. Biol.* 14: 949 (1990); Yamaguchi-Shinozaki et al., *loc. cit.* 15: 225 (1990); Kuhlemeier et al., *Plant Cell* 1: 471 (1989).

The gene encoding a foreign protein may be expressed in a tissue-specific or developmentally regulated manner. For example, the gene may be operably linked to a promoter which is specifically expressed in pollen, root or seed. For example, see Albani et al., *Plant Mol. Biol.* 16: 501 (1991);, Depater et al., *loc. cit.* 18: 161

(1992); and Knutzon et al. *Proc. Nat'l Acad. Sci. U.S.A.* 89: 2624 (1992).

Mitotic stability can be achieved using plant viral vectors that provide epichromosomal replication.

- 5 An alternative and preferred method of obtaining mitotic stability is provided by the integration of expression vector sequences into the host chromosome.

4. Transformation Methods

- 10 Biological and physical plant transformation protocols are available.

A. *Agrobacterium*-mediated Transformation

- The most widely utilized method for introducing an
15 expression vector into plants is based on the natural transformation system of *Agrobacterium*. See, for example, Horsch et al., *Science* 227: 1229 (1985). *Agrobacterium tumefaciens* and *A. rhizogenes* are plant-pathogenic soil bacteria which genetically transform
20 plant cells. The Ti and Ri plasmids of *A. tumefaciens* and *A. rhizogenes*, respectively, carry genes responsible for genetic transformation of the plant. See, for example, Kado, *Crit. Rev. Plant. Sci.* 10: 1 (1991). Descriptions of *Agrobacterium* vector systems and methods
25 for *Agrobacterium*-mediated gene transfer are provided by Gruber et al., *supra*, Miki et al., *supra*, and Moloney et al., *Plant Cell Reports* 8: 238 (1989).

B. Direct Gene Transfer

- 30 Several methods of plant transformation, collectively referred to as direct gene transfer, have been developed as an alternative to *Agrobacterium*-mediated transformation. Microprojectile-mediated; sonication; liposome or spheroplast fusion; direct uptake of DNA into
35 protoplasts using CaCl_2 precipitation, polyvinyl alcohol or poly-L-ornithine; and electroporation of protoplasts and whole cells are described, *supra*.

5. Promoters

A. Inducible Promoters

Exemplary inducible promoters that can be utilized in the instant invention include that from the ACE1 system which responds to copper (Mett et al. *PNAS* 90: 4567-4571 (1993)); In2 promoter from maize which responds to benzenesulfonamide herbicide safeners (Hershey et al., *Mol. Gen. Genetics* 227: 229-237 (1991), and Gatz et al., loc. cit. 243: 32-38 (1994)) or Tet repressor from Tn10 (Gatz et al., *ibid.* 227: 229-237 (1991)). A promoter that responds to an inducing agent to which plants do not normally respond can be utilized. An exemplary inducible promoter is the inducible promoter from a steroid hormone gene, the transcriptional activity of which is induced by a glucocorticosteroid hormone. Schena et al., *Proc. Nat'l Acad. Sci. U.S.A.* 88: 10421 (1991)

B. Tissue-specific or Tissue-Preferred Promoters

Exemplary tissue-specific or tissue-preferred promoters that can be utilized in the instant invention include a seed-preferred promoter such as that from the phaseolin gene (Murai et al., *Science* 23: 476-482 (1983), and Sengupta-Gopalan et al., *Proc. Nat'l Acad. Sci. USA* 82: 3320-3324 (1985)); a leaf-specific and light-induced promoter such as that from *cab* or *rubisco* (Simpson et al., *EMBO J.* 4: 2723-2729 (1985), and Timko et al., *Nature* 318: 579-582 (1985)); an anther-specific promoter such as that from *LAT52* (Twell et al., *Mol. Gen. Genet.* 217: 240-245 (1989)); a pollen-specific promoter such as that from *Zm13* (Guerrero et al., *Mol. Gen. Genet.* 224: 161-168 (1993)); and a microspore-preferred promoter such as that from *apg* (Twell et al., *Sex. Plant Reprod.* 6: 217-224 (1993)).

35 C. Constitutive Promoters

Exemplary constitutive promoters that can be utilized in the instant invention include the promoters from plant viruses such as the 35S promoter of CaMV (Odell et al.,

Nature 313: 810-812 (1985)) and the promoters from such genes as rice actin (McElroy et al., Plant Cell 2: 163-171 (1990)); ubiquitin (Christensen et al., Plant Mol. Biol. 12: 619-632 (1989) and Christensen et al., Plant Mol. Biol. 18: 675-689 (1992)); pEMU (Last et al., Theor. Appl. Genet. 81: 581-588 (1991)); MAS (Velten et al., EMBO J. 3: 2723-2730 (1984)) and maize H3 histone (Lepetit et al., Mol. Gen. Genet. 231: 276-285 (1992) and Atanassova et al., Plant Journal 2(3): 291-300 (1992)).

The ALS promoter, a XbaI/NcoI fragment 5-prime to the *Brassica napus* ALS3 structural gene (or a nucleotide sequence that has substantial sequence similarity to said XbaI/NcoI fragment), represents a particularly useful constitutive promoter. The ALS promoter is described in co-pending U.S application serial No. 08/409,297, which is incorporated herein by reference.

6. Signal Sequences For Targeting Proteins to Subcellular Compartments

Transport of GFP or other proteins to a subcellular compartment such as the chloroplast, vacuole, peroxisome, glyoxysome, cell wall or mitochondrion, or for secretion into the apoplast, is accomplished by means of operably linking the nucleotide sequence encoding a signal sequence to the 5-prime and/or 3-prime region of a gene. Targeting sequences at the 5-prime and/or 3-prime end of the structural gene may determine, during protein synthesis and processing, where the encoded protein is ultimately compartmentalized. The presence of a signal sequence directs a polypeptide to either an intracellular organelle or subcellular compartment or for secretion to the apoplast.

Exemplary signal sequences that can be utilized in the instant invention include chloroplast signal sequences (Becker et al., Plant Mol. Biol. 20: 49 (1992)); mitochondrial signal sequences (Close, P.S., Master's Theses, Iowa State University (1993)); the barley alpha amylase 1 signal sequence (Knox et al.,

Plant Mol. Biol. 9:3-17 (1987)); the barley lectin vacuole signal sequence (Lerner et al., *Plant Physiol.* 91: 124-129 (1989)); the endoplasmic reticulum retention signal (Fontes et al., *Plant Cell* 3: 483-496 (1991)); the
5 sweet potato sporamin vacuole signal sequence (Matsuoka et al., *Proc. Nat'l Acad. Sci.* 88: 834 (1991)); the consensus peroxisome signal sequence (Gould et al., *J. Cell Biol* 108: 1657 (1989)); and the pea glutathione reductase mitochondrial and chloroplast signal sequence
10 (Creissen et al., *Plant J.* 2: 129 (1991)).

Example 1

15 *Isolation of Unfertilized and Fertilized Embryo Sacs from Maize*

Plants of *Zea mays* L. (cultivar CV129) were grown to maturity in growth rooms at the University of Alberta.
20 At anthesis, pollen was collected by shaking the tassels over a tray. The collected pollen was used immediately for hand-pollination.

Plants used for cob production were placed in a pollen-free environment by removing plants from the
25 pollen-producing rooms prior to silk emergence. The plants were emasculated, washed to remove any surface pollen and maintained in a pollen-free room for cob development. Emerging cobs were bagged to prevent any indiscriminate pollination.

30 Embryo sacs containing unfertilized egg cells were isolated from plants having silks measuring approximately 6 cm in length. The length of the silk is not critical, however, to isolation of viable embryo sacs. As long as the silk is not pollinated it can grow to any length
35 including from 1 to 40 cm in length. Silk length depends on such factors as maize genotype or growth conditions.

Embryo sacs containing fertilized eggs cells were also isolated from plants having silks measuring 6 to 10

cm in length. Once again, however, silk length is not critical to isolation of viable embryo sacs. Plants were removed from the pollen-free room and hand-pollinated with fresh pollen. Cobs were subsequently bagged and the plants were placed in a growth chamber for fertilization to occur. Fertilized embryo sacs were isolated at 16 or more hours after pollination. However, the duration between pollination and embryo sac isolation can be reduced. If plants with shorter silks are used or if the husk leaves are pulled back to expose the silks close to the ovules before applying pollen, fertilized embryo sacs can be isolated as soon as 3-5 hours after pollination.

Unfertilized or fertilized embryo sacs were isolated by removing husks and silks from the cobs. The cobs were cut transversely into approximately 3 cm segments and the segments were surface sterilized for 10 minutes in 70% ethanol followed by three rinses in sterilized, distilled and deionized water. Ovaries were removed and mounted for sectioning.

Specimen blocks for use in the microtome were surface sterilized in 70% ethanol for 10 minutes. The blocks were placed in a row in a laminar flow hood. After evaporation of the alcohol, a thin layer of a fast-acting adhesive, "Quick Set 404" (Loctite Corporation, Newington, CT), was applied to the top of each block and the ovaries were placed in the proper position. The ovaries were removed in pairs, with two or three pairs placed on each block. The ovaries were placed with their adaxial surface up and perpendicular to the long axis of the specimen block as shown in Figure 1.

The blocks with the attached ovaries were placed in the Vibratome (Technical Products International, St. Louis, MO) with the stylar ends of the ovaries facing the blade as shown in Figure 2. The blade was positioned above the ovaries and the specimen block was raised to begin sectioning. The ovaries were sectioned at a thickness of 200 to 400 μm . The first block was serially sectioned in 200 μm increments from the adaxial surface

of the ovaries. Every section was then observed to determine the approximate distance of the embryo sac from the surface. Once the approximate distance of the embryo sac from the surface was determined, sectioning was commenced with the goal of slicing on either side of the embryo sacs but not through them. Thinner sections may improve embryo sac visibility but result in damage to many embryo sacs. Thicker sections result in more intact embryo sacs but with reduced resolution, due to the thickness of the supporting nucellus. Section thicknesses of 250 to 300 μm routinely produce numerous, viable embryo sacs with acceptable visibility. The sections containing the embryo sacs were collected and placed on a modified MS medium with 0.4 mg/L L-asparagine, 0.1 mg/L 6-benzylaminopurine (BAP) and 15% sucrose, pH 5.8. Murashige et al., *Physiol. Plant* 15: 473 (1962); Campenot et al., *Amer. J. Bot.* 79: 1368 (1992).

The sections were assessed by observing both sides using a stereomicroscope with basal illumination. A section can be easily manipulated by grasping the ovary wall with fine forceps. Intact embryo sacs were placed with their most visible side facing upward for microinjection.

25

Example 2

Microinjection of Maize Zygotes in Isolated Embryo Sacs

Embryo sacs containing zygotes were isolated as described *supra*. Maize zygotes in isolated embryo sacs were transformed with plasmid PHP3953 which is shown in Figure 3. Plasmid PHP3953 carries the GUS structural gene operably linked to the maize ubiquitin (Ubi-1) promoter. Plasmid DNA (100 μg) was vacuum-dried in Tris-EDTA buffer and dissolved in 0.5 ml milli-Q water. The resulting preparation was divided into 10 μl aliquots and stored at -20°C until use.

35

Microinjection pipettes were pulled from borosilicate

tubing (1.0 mm x 0.75 mm, Sutter Instrument Co.) on a micropipette puller (P-97, Sutter Instrument Co.), bevelled to a tip diameter of 1 to 5 μ m with a pipette grinder (BV-10, Sutter Instrument Co.) and autoclaved.

5 After being loaded with plasmid using a microloader (Eppendorf), the microinjection pipette was connected to a Transjector 5246 (Eppendorf). The injection unit of the Transjector was mounted on a MMN-1 three-dimensional manipulator (Narishige) on a Stemi SV11 Stereomicroscope

10 (Zeiss). The angle between the injection pipette and the section was about 45°. The microscope that supports the micromanipulator and injection unit was housed in a laminar flow hood.

The tip of the injection micropipette was brought to the surface of sections by means of a micromanipulator.

15 The tip of the injection micropipette was brought just above the target cells, either the zygote or the central cell. Penetration of a cell was achieved using an hydraulic joystick. Injection volume was adjusted with injection pressure and injection time to about 1 pl.

20 Approximately 100 to 120 embryo sacs can be injected per hour.

GUS activity was determined 5 days after microinjection using a standard GUS histochemical staining protocol. Jefferson, R.A., *Plant Mol. Biol. Rep.* 5: 3-7 (1987). By that time, endosperm growth was apparent. The embryo sacs were incubated in a petri dish containing a solution comprised of 0.1 M phosphate buffer (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5

25 mM potassium ferrocyanide, 0.1% triton-X 100 and X-gluc (1 mg/ml) for 30 min under vacuum. The embryo sacs were transferred to an incubator at 37°C. Specimens were examined with the stereomicroscope one hour after incubation.

35 Histochemical sections from GUS-positive embryo sacs were fixed in glutaraldehyde to determine the cellular location of GUS expression in the embryo sacs. The embryo sacs were fixed overnight in 2% glutaraldehyde.

After dehydration in alcohol, the specimens were embedded in Spurr's resin and examined with a brightfield or differential interference contrast microscope. Spurr, A.R., *J. Ultrastruct. Res.* 26: 31 (1969).

5 Within one week of culture approximately 71% of the isolated embryo sacs produced endosperm. The remaining embryo sacs which failed to develop endosperm only rarely produced an embryo. Embryos developed in approximately 60% of the embryo sacs that produced endosperm.

10 GUS was detected in approximately 10% of the microinjected embryo sacs 5 days after microinjection of the zygote with PHP3953. The GUS-positive embryo sacs were blue in color. Microscopic analysis of GUS-positive embryo sacs revealed that both zygotes and endosperm
15 appear to be transformed.

 Using a similar microinjection method and DNA concentration, plasmid PHP8088 is injected into zygotes. Plasmid PHP8088 is shown in Figure 4 and carries the GFP structural gene operably linked to the maize Ubi-1
20 promoter. The embryo sacs are screened for detectable GFP with a Carl Zeiss microscope containing a lamp housing with a 75W xenon burner (38-00-53) equipped with a high Q interference exciter filter and a green barrier filter. A fiber optic gooseneck is utilized for scanning
25 petri dishes for GFP-containing cells.

Example 3

Recovery and Characterization of Transgenic

30

Plants Produced from Transformed Zygotes

 Following microinjection of zygotes with PHP3953, embryo sacs were cultured at 25°C for 5 days in the dark
35 on modified MS medium containing 0.4 mg/L L-asparagine, 0.1 mg/L 6-benzylaminopurine (BAP) and 15% sucrose at pH 5.8. Campenot et al., *Amer. J. Bot.* 79: 1368 (1992). Endosperm enlargement was observed after 5 days in culture.

Embryo sacs were then transferred to modified MS medium containing 0.4 mg/L L-asparagine and 10% sucrose at pH 5.8 and no BAP. Following incubation for another 5 days in the dark, embryos were transferred to modified MS medium containing 0.4 mg/L L-asparagine and 3% sucrose at pH 5.8 and no BAP. When young shoots were approximately 1.5 cm long they were exposed to light.

Five days after exposure to light, root tips measuring 1 to 2 cm in length were excised from each seedling for histochemical assay of GUS activity. After excision of root tips seedlings were transferred to a nutrient solution at pH 6.5. Zhang et al., *Plant Physiol.* 94: 577 (1990).

Roots excised from microinjected plants and control plants were incubated in the GUS assay mixture at 37°C under aseptic conditions. Samples were examined after several hours of incubation with a Stemi SV11 Stereomicroscope (Zeiss). Intact roots from control plants were light brown in color with no evidence of GUS activity. Roots from control plants were squashed under a cover slip and observed under the Zeiss compound microscope. No GUS activity was observed in the root tissue including the cortex and procambium.

GUS activity was detected in the roots excised from plants recovered from embryo sacs microinjected with PHP3953. Three cases of transformants were observed; specifically, blue colored cells were observed in (1) the cortex, (2) the procambium, and (3) the cortex and procambium. None of the transgenic plants obtained through microinjection of zygotes exhibited abnormalities.

Example 4

Isolation of Zea mays Sperm Cells

Three discontinuous Percoll gradients were prepared for each gram of pollen collected. The Percoll was filter sterilized through a sterile 10 cc syringe with a

Millex-GS filter. This filter was placed on the end of the syringe together with a 18G1/2 needle. The discontinuous gradients were made in 15 ml corex test tubes with round bottoms. Approximately 2 to 3 ml of sterile 30% Percoll in Gal-Mes buffer (0.55 M D-galactose; 2 mM 2-(N-morpholino)ethanesulfonic acid; pH 6.7)) was placed in each corex tube. (Sufficient 30% Percoll must be in the tube to avoid disturbing the pellet during sperm cell removal.)

10 Approximately 2 to 3 ml of sterile 15% Percoll in Gal -Mes buffer (pH 6.7) was carefully placed on top of the 30% Percoll. The sperm cells were found at the interface of the 30% and 15% Percoll. Finally, 2 ml of sterile 10% Percoll in Gal-Mes buffer (pH 6.7) was placed
15 on top of the 15% Percoll. The 10% Percoll was required to remove some of the smaller particles from the sperm cells.

 Pollen was collected in a flat tray at anthesis by shaking the tassels. The collected pollen was utilized
20 immediately to avoid desiccation. The pollen grains were separated from the anthers by means of a soft paint brush; the tray was gently shaken during this process.

 Fresh pollen was separated by collecting the fresh pollen on the surface of 80 μ m nylon mesh. Only 2 to 3
25 grams of freshly collected pollen was placed on the 5-inch square piece of nylon mesh in order to increase the efficiency of separation. The mesh was shaken gently so that the wrinkled pollen that is not fresh was removed. The fresh pollen collected on the surface of the mesh was
30 placed in a petri dish which was then inserted into a hydration chamber.

 The pollen was hydrated for no more than 20 minutes in a covered glass jar containing water in the bottom. The pollen was then osmotically disrupted in a 50 ml
35 Erlenmeyer flask containing 15 ml of Gal-Mes buffer per gram of pollen. The flask was shaken for approximately 20 minutes. Under these conditions the pollen grains burst releasing sperm cells. This mixture was filtered

through a 40- μ m stainless steel mesh filter with suction to reduce filtering time in order to separate intact pollen and debris from the sperm cells.

5 A 10 ml syringe with a 18G1/2 needle was used to deliver the filtrate to a discontinuous Percoll gradient as described above. The sperm cells appeared as a smooth white/yellow band at the boundary between the 15% and 30% Percoll layers. The sperm cells were collected with a sterile pipette and placed in a sterile conical tube.
10 Fresh sterile Gal-Mes (pH 6.7) was added to the sperm cells in a ratio of at least 2 ml of gal-MES per ml of sperm cell solution. Following mixing the tubes were centrifuged at 3000 g for 10 minutes and the sperm cells pelleted at the bottom of the tube. The sperm cells were
15 resuspended in Gal-Mes buffer (pH 6.7) at the desired concentration. One gram of maize pollen yielded approximately 3 to 4 million sperm cells using the method described above.

20

Example 5

Transformation of Sperm Cells and Delivery of Foreign Gene to the Egg Cell

Maize sperm cells are isolated from freshly collected
25 pollen according to the method described *supra*. Final resuspension is performed in 10% Gal-Mes buffer (pH 6.7). Cells are diluted to approximately 2.0×10^6 cells per ml and 150 μ l placed in a 0.4 cm gap electroporation cuvette. KCl is added to a final concentration of 75 mM
30 together with a rhodamine dextran solution (50 μ l of a 15 mg per ml stock solution). The suspension then is subjected to electroporation at 250 V and 250 μ F and then incubated at room temperature for 10 min to allow resealing of the cell membranes. The presence of the
35 rhodamine label is detected by both conventional and confocal laser microscopy.

The sperm cells are transformed with a vector that carries one or more foreign genes of interest as well as a detectable marker gene. For example, the vector may

carry a gene encoding GFP. Alternatively, the vector may carry a fluorescent label. Sperm cells transformed with the vector are separated from non-transformed cells, for example, by flow cytometry and cell sorting. The fusion
5 of sperm and egg cells is facilitated by means of electrical or chemical stimuli. See Kranz et al., supra and Deprez et al., supra.

Example 6

10 Isolation of Nuclei from Transformed Sperm Cells

Sperm cells are isolated and transformed with a foreign gene as described, supra. Transformed sperm cells are suspended in 1 ml Gal-Mes buffer (pH 6.7) and vortexed following addition of 9 ml of 2mM Mes (ph 6.7)
15 and 0.1 mM KNO₃ to rupture the plasma membrane. The osmolarity of the suspension was reestablished by addition of 2 ml 50% galactos in 2 mM Mes (ph 6.7). The mixture containing nuclei and cell debris is loaded on Percoll gradients containing 70, 50 and 30% Percoll in
20 Gal-Mes buffer (pH 6.7). The gradients are centrifuged at 2,000xg for 15 min. Sperm nuclei are collected at the interface between the 50 and 70% Percoll layers, washed with 5 ml Gal-Mes buffer and centrifuged at 1,000xg for 10 min. Pellets containing sperm nuclei are resuspended
25 in 100 μ l Gal-Mes buffer. Isolated sperm nuclei are introduced into egg of isolated embryo sacs by microinjection.

Example 7

Use of *Agrobacterium* for Zygote Transformation

Embryo sacs containing zygotes are isolated as described *supra*. Maize zygotes in isolated embryo sacs
5 are transformed using *Agrobacterium tumefaciens* strain LBA4404. See Hiel et al., *The Plant Journal* 6: 271-282 (1994). For *Agrobacterium*-mediated transformation of maize, super-virulent plasmids similar to those described by Hiel et al., *supra* and Ishida et al., *supra* are used.
10 For example, the backbone of PHP8904 is a pTOK246-based vector with the origin of replication of pBR322, a spectinomycin-resistance gene and the T-DNA borders. See Ishida et al., *supra*.

Two plant expression units encoding GUS and
15 phosphinothricin-acetyl-transferase (PAT) enzymes are cloned between the right and left border sequences. The first expression unit consists of the maize ubiquitin promoter and the ubiquitin intron; the uidA gene interrupted within the structural gene by a potato
20 catalase intron; and the potato 3' pin II sequence. Christensen et al., *Plant Mol. Biol.* 18: 675-689 (1992); Jefferson et al., *EMBO J.* 6: 3901-3907; Vancanneyt et al., *Mol. Gen. Genet.* 2290: 245-250 (1990); An et al., *Plant Cell* 1: 115-112 (1989).

25 The second expression unit contains the double 35S promoter; the omega prime (O') 5-prime sequence; the adh1 intron; the bar structural gene; and the pin II 3-prime sequence. Close, P., Ph.D. Dissertation, Iowa State University; Gallie et al., *Nucl. Acid Res.* 15: 3257-3273
30 (1987); Dennis et al., *loc. cit.* 12: 3983-3990 (1984); and White et al., *ibid.* 18: 1062 (1990).

Agrobacterium is grown and used in a manner similar to that described in Ishida et al., *supra*. LBA4404 (PHP8904) is grown for 3 days on YP medium (5 g/l yeast
35 extract, 10 g/l peptone, 5 g/l NaCl, 15 g/l agar, pH 6.8) supplemented with 50 mg/l spectinomycin. Bacteria are collected with a loop from the solid medium and resuspended. For *Agrobacterium* treatment of isolated

embryo sacs, the bacteria are resuspended in the modified MS-based medium used for zygote culture. The concentration of *Agrobacterium* used for infection and co-cultivation may need to be varied. Infection is initiated by placing the nucellar sections in the *Agrobacterium* suspension. After about 30 minutes the sections are removed from the liquid and placed on solid zygote culture medium with no antibiotics for the co-cultivation period. It is understood in the art that high densities and long co-cultivation can injure maize cells. Thus, a range of *Agrobacterium* concentrations from 10^5 - 10^{10} cells/ml and a range of co-cultivation periods from a few hours to 3 days are tested with the nucellar sections containing the embryo sacs.

In this manner, the optimal conditions for DNA introduction and zygote survival and development can be found. After the co-cultivation period, the maize tissue is transferred onto zygote culture medium with antibiotics such as 250 mg/l cefotaxime or 100 mg/l carbenicillin. Embryo and plant development continue on antibiotic-containing media. Plants are assayed for the presence and expression of foreign genes such as uidA and PAT using methods described *supra*. Reciprocal crosses are made between non-transgenic plants and T0 plants identified as transgenic. Alternatively, transgenic plants are self-pollinated in order to pass the transgenic trait to progeny.

Example 8

Microinjection of Sperm Cells Into Isolated Embryo Sacs

Unfertilized maize embryo sacs were isolated according to the method described in Example 1. Maize sperm cells were isolated according to the method described in Example 4. Sperm cells were microinjected into exposed embryo sacs by inserting the micropipette just above (chalazal to) the egg apparatus according to the method described in Example 2. The egg cells were clearly visible and in many cases the 2 synergids could

also be seen. The volume of fluid injected was adjusted to 250-300 pL and each injection contained 2 sperm cells.

Following microinjection, the embryo sacs were
5 cultured as described in Example 3. Endosperm development was observed in 5 days after microinjection. The endosperm was 1.5 mm in length 14 days after microinjection. Microscopic observation of the stained samples revealed that the embryo sacs
10 microinjected with sperm cells developed fully cellular endosperm. Additionally, embryo sacs microinjected with sperm cells contained developing embryos in the micropylar portion of the embryo sac. These embryos underwent normal tissue and organ development.

15 To determine ploidy level, microinjected embryo sacs were embedded in paraffin or plastic by Campenot et al., A. J. Bot 79(2):1368-1373 (1992). DNA content was determined based on quantitative microphotometry described by Mogensen, H.L. and Holm, P.B., *Plant Cell*
20 7: 487-494 (1995). Specimens were fixed in 95% ethanol and glacial acetic acid (3:1) for 30 min. After dehydration in 95% ethanol for 30 min. and in absolute ethanol for 3 min., specimens were stained in 1 μ g mL⁻¹ 4',6-diamidino-2-phenylindole (DAPI) for 1 h and
25 mounted with Aquamount. Fluorescence intensity was measured using an Axiovert 135 inverted microscope (Zeiss) equipped with UV light from a 100 watt Hg/Xe-arc lamp, excitation filter 330-380 and barrier filter BA420 and an intensified charged coupled device (CCD)
30 camera (Paultek Imaging). Fluorescence images were digitized and stored using a Macintosh Quadra 950 computer equipped with a Quickcapture frame grabber board (Data Translation). Fluorescence intensity was analyzed using NIH Image (1.56).

35 Quantitative analysis of ploidy level by microphotometry revealed that the unfertilized embryos microinjected with sperm cells according to the method described supra produced embryos that were 2N and

endosperm that was 3N. Accordingly, microinjection of unfertilized embryo sacs with sperm cells according to the method described *supra* resulted in normal embryo and endosperm development.

5

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following claims.

15 All publications and patent applications mentioned in this specification are indicative of the level of skill of those in the art to which the invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if
20 each individual publication or patent application were specifically and individually indicated to be incorporated by reference in its entirety.

What Is Claimed Is:

1. A method for producing a transformed plant, comprising the steps of (a) isolating an embryo sac from a plant; (b) introducing an expression vector carrying a foreign gene into the zygote or embryo in said embryo sac; and then (c) recovering a transformed plant from said zygote or embryo.

2. A method according to claim 1, wherein step (b) comprises (i) introducing an expression vector carrying said foreign gene into the egg cell of said isolated embryo sac; and then (ii) fertilizing said transformed egg cell with a plant sperm cell isolated from said plant, to produce said zygote or embryo.

3. A method according to claim 1, wherein step (b) comprises fertilizing the egg cell in said isolated embryo sac with a plant sperm cell that contains an expression vector carrying a foreign gene, to produce said zygote or embryo.

4. A method according to claim 1, wherein step (b) comprises (i) introducing an expression vector carrying a foreign gene into sperm cells from a plant; (ii) isolating nuclei from said transformed sperm cells; and (iii) fertilizing the egg cell in said isolated embryo sac with said isolated nuclei, to produce said zygote or embryo.

5. The method according to any of claims 1, 2, 3 and 4, wherein said plant is a monocot.

6. The method according to claim 5, wherein said plant is a cereal.

7. The method according to claim 6, wherein said plant is maize.

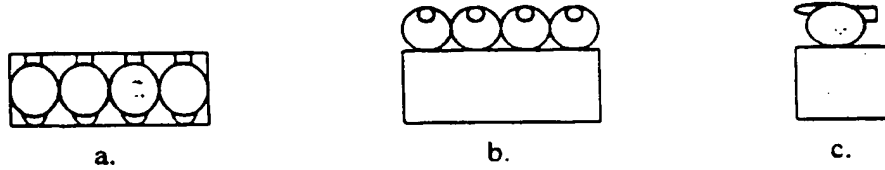
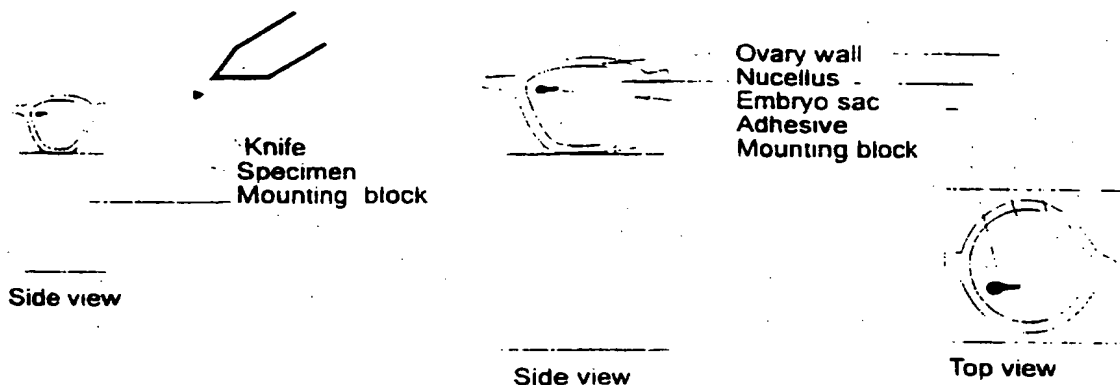


Fig. 1. Orientation of the ovaries on the specimen blocks prior to sectioning.
a) Top view. b) Front view and c) Side view.

Fig. 2. Isolation of Maize Embryo Sacs using the Vibratome

A) Components



B) Method

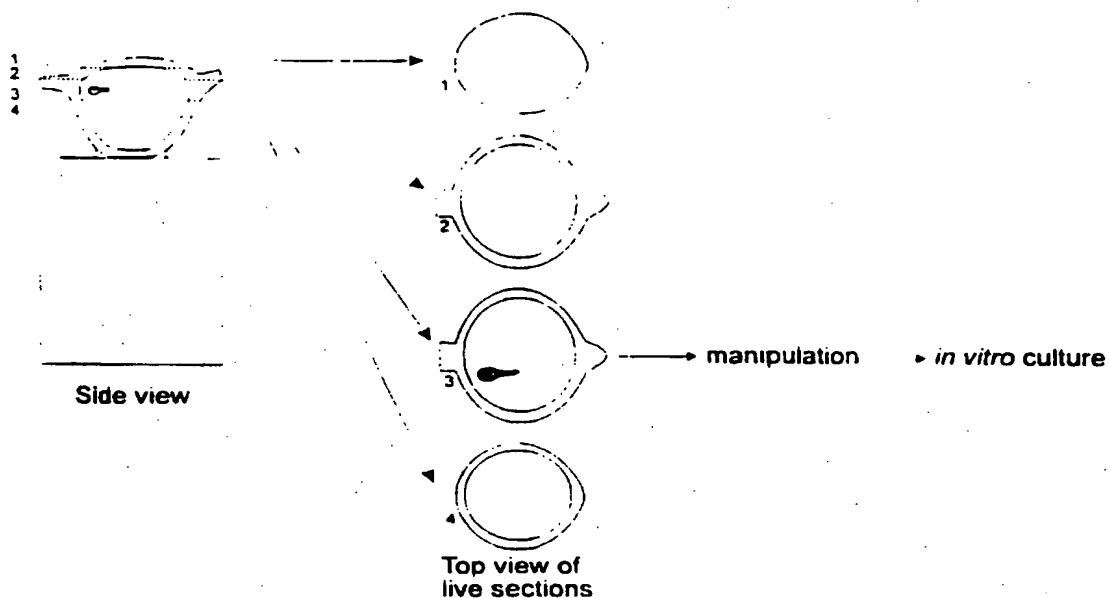


Figure 3

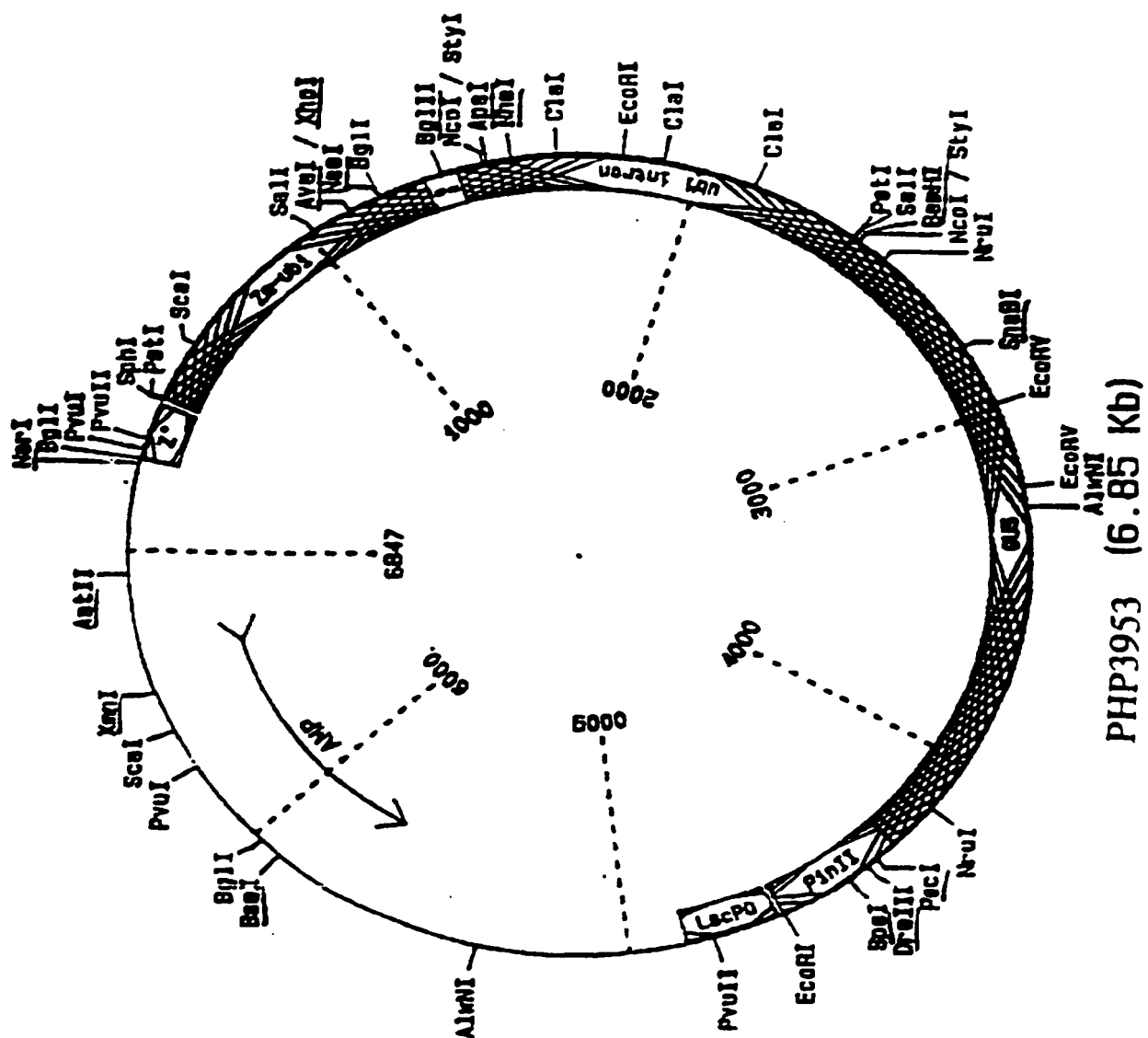
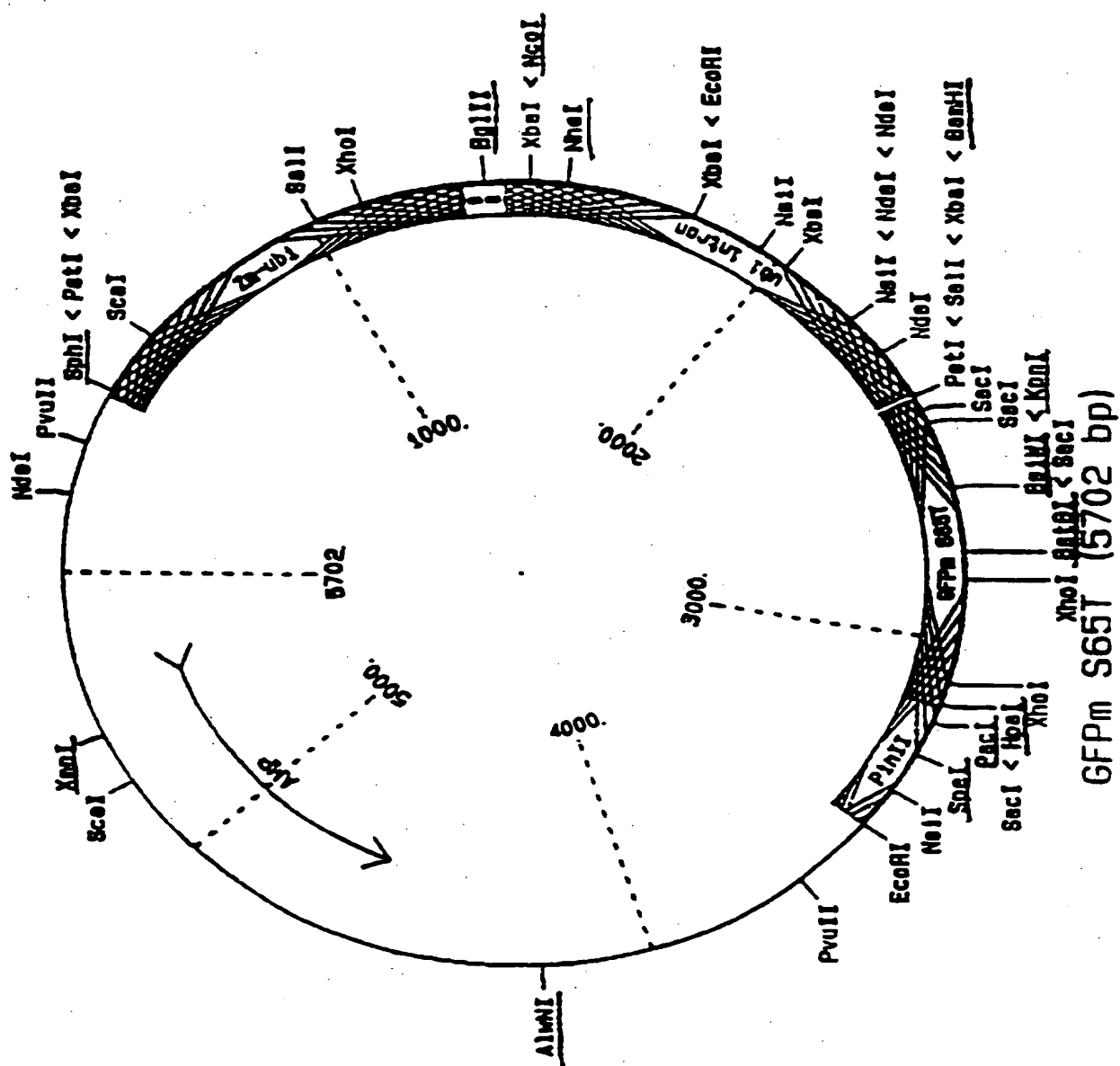
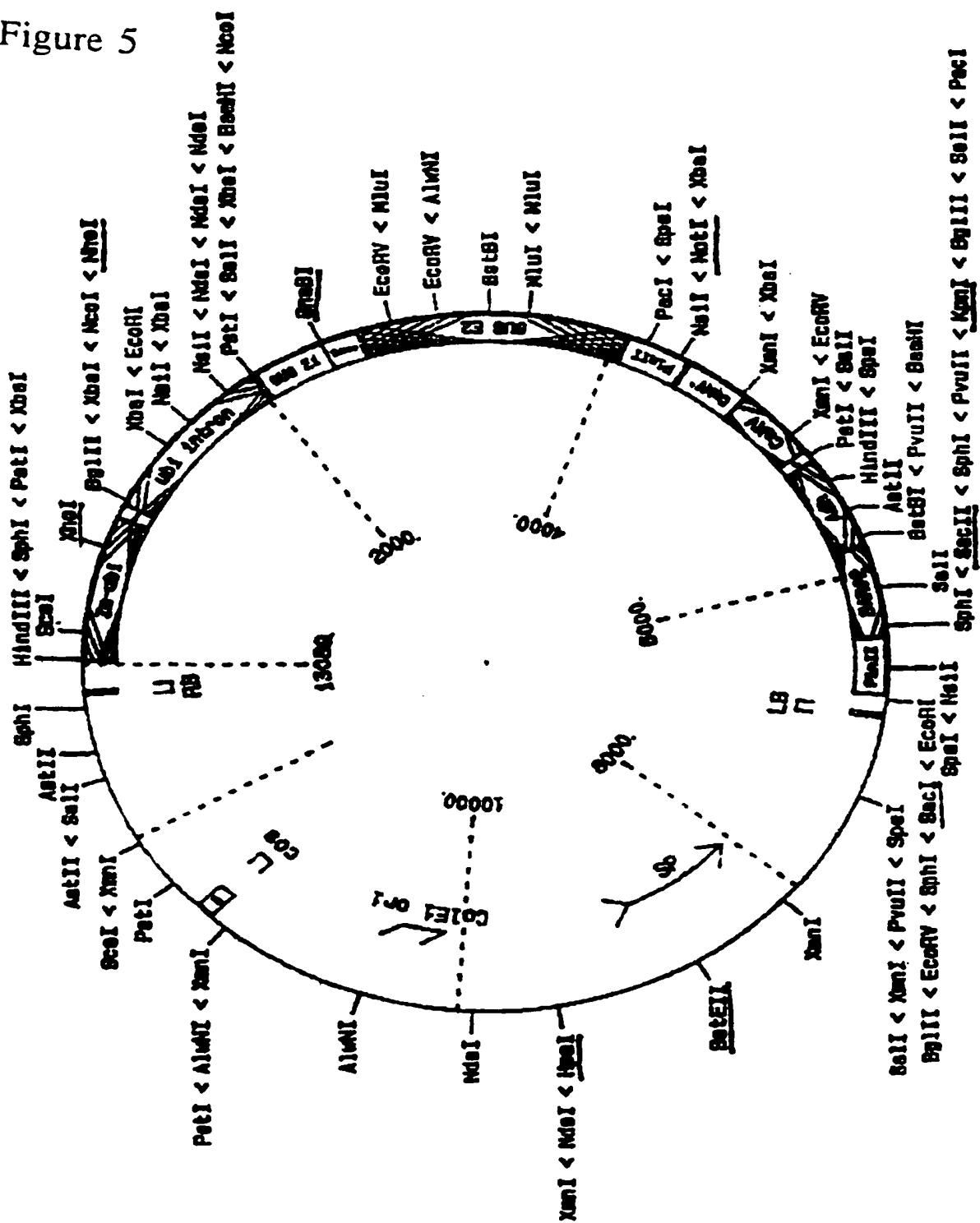


Figure 4



PHP8904 (13,089 bp)

Figure 5



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/11184

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 331 083 A (SCHWEIZ EIDGENOSSENSCHAFT) 6 September 1989 see the whole document	1,5-7
X	ISHIDA, Y., ET AL.: "High efficiency transformation of maize (Zea mays L.) mediated by Agrobacterium tumefaciens" BIOTECHNOLOGY, vol. 14, no. 6, June 1996, pages 745-750, XP002046364 see the whole document	1,5-7
Y	WO 94 00583 A (SOUTH DAKOTA STATE UNIVERSITY) 6 January 1994 see the whole document	1-7
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

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- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- *Z* document member of the same patent family

Date of the actual completion of the international search

17 November 1997

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/11184

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Y	HAHN, S.K., ET AL.: "Biotechnology in reproductive biology" ANGIOSPERM POLLEN AND OVULES, 1992, pages 340-346, XP002046592 see page 345, last paragraph ---	2
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International Application No
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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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PCT/US 97/11184

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